CHAPTER 3:
 STUDY PLAN AND PROCEDURE
3.1. Study Design:

i) The study was designed to obtain data on cardio metabolic risk profile and anaemia and their correlates in both pre and post menopausal Indian women comprising of subjects from two different ethnic and nonethnic communities.

ii) Ethical clearance for the study was obtained from Institutional Human Ethical Committee before onset of the study.

iii) Informed consent from all participating subjects were obtained before including them in the study.

3.2 Study population:

i) The study was conducted on apparently healthy subjects accompanying the patients attending the OPDs of Shri B.M. Patil Medical college, Vijaypur, Karnataka, and Tripura Medical College, Agartala, Tripura, representing both urban and rural subjects from a mixed Indian population. The subjects included women from two non ethnic and two ethnic groups of India in this Hospital based observational study. The groups are as follows:

   a) Non ethnic subjects from Vijaypur, Karnataka.
   b) Non ethnic subjects from Agartala, Tripura.
   c) Ethnic Lambanis from Vijaypur, Karnataka, India.
   d) Ethnic Riang tribe of Tripura, a North eastern state of India, and

ii) Age of the subjects varied from 25 to 65 years.
3.2.a. Sample size:

As the reported prevalence of cardio metabolic risk varies from 10% to 50% in Indian subjects from both sexes and all age groups, a prevalence of 30% was taken to calculate the sample size for a mixed Indian population with 95% interval and absolute precision of 5%. The formula for sample size calculation as mentioned in WHO Manual for Sample size calculation in Health studies was used to calculate the required sample size for the study. The formula was: \(4PQ/L^2\), where \(P=\) Prevalence (30%), \(Q=(1-P)\), \(L=\) Confidence interval (95%), Maximum allowable error=0.05. Thus the minimum sample size estimated was 323 (Table - Ia, page 25 of the Manual). The final sample size (335), however, was higher than this number.

3.2.b. Inclusion criteria:

Only healthy women who were non-smokers, non-alcoholics, nonusers of hormonal contraceptives and hormone replacement therapy were included for the study.

3.2.c. Exclusion criteria:

i) Known diabetics.

ii) Known hypertensives.

iii) Subjects on lipid lowering drugs.

iv) Subjects with history of polycystic ovary or any other chronic diseases.

v) Subjects with other endocrine disorders (Thyroid, Adrenal).
3.2.d. Flow of the subject:

Figure I: Flow of study subjects.
3.2.e. Distribution of subjects from different Ethnic groups:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Premenopausal</th>
<th>Postmenopausal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non ethnic subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from Agartala, Tripura.</td>
<td>61</td>
<td>36</td>
<td>97</td>
</tr>
<tr>
<td>Non ethnic subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FromVijaypur, Karnataka</td>
<td>47</td>
<td>32</td>
<td>79</td>
</tr>
<tr>
<td>Ethnic Riang subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From Tripura</td>
<td>59</td>
<td>33</td>
<td>92</td>
</tr>
<tr>
<td>Ethnic Lambani subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FromVijaypur, Karnataka</td>
<td>36</td>
<td>31</td>
<td>67</td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>132</td>
<td>335</td>
</tr>
</tbody>
</table>

**Table II**: Distribution of subjects from different ethnic groups.
3.3. STUDY PROTOCOL:

i) The subjects were first informed about the purpose of the study and the procedures involved. Once they volunteered for the study, the detailed procedure was explained to the subjects as outlined in the informed consent. Informed consent was taken from the volunteered subjects (ANNEXURE - I).

ii) At the onset of the study, a proforma was filled to evaluate general health status of the subject, history of past illness, information regarding menstrual and marital status, history of pregnancy along with personal and family history (ANNEXURE -II).

iii) Women who were still menstruating irrespective of regularities of their menses were considered as premenopausal women, while postmenopausal women were those women who had ceased menstruation for at least one year. 

iv) In the second stage, various anthropometric and physiological parameters of the subjects were recorded.

v) In the third and final stage, venous blood was collected from each subject for analysis of various haematological and biochemical parameters.

vi) All the recordings were entered in a proforma (ANNEXURE - III)

3.3. a. Study period:

Study was conducted between November 2012 to October 2014.

3.3.b. RECORDING OF ANTHROPOMETRIC PARAMETERS:

Anthropometric parameters recorded in the subjects were:

- Height (cm), Body Weight (kg), Waist Circumference (cm), Hip Circumference (cm), Body Mass Index (BMI) (kg/m²), Waist -Hip Ratio (WHR), and Waist -Height Ratio (WHtR).

- Height of the subject was recorded by using a stadiometer with subject standing erect.

- Weight of the subject was recorded with subject standing erect on a human weighing machine in light clothing.

- Waist circumference was measured at midpoint between the last rib and iliac crest by using a measuring tape.
Hip circumference was measured at the widest level over the greater trochanters to nearest centimetres by using a measuring tape.

BMI was calculated as Kg/m². WHR and WHtR were also calculated.

3.3.c. RECORDING OF BLOOD PRESSURE AND HEART RATE:

Heart Rate (HR) of the subject was recorded as beats/min in sitting posture.

Both systolic and diastolic blood pressures were recorded in each subject by using a sphygmomanometer in sitting posture. Two recordings were taken for each subject with a gap of five minutes. Average of three measurements was used in the analysis. Pulse Pressure (PP) was calculated as difference of systolic and diastolic blood pressure.

3.3.d. COLLECTION OF BLOOD SAMPLES FOR HEMATOLOGICAL AND BIOCHEMICAL ANALYSIS:

Venous blood sample (10 ml) was drawn from each individual after an overnight fasting for haematological and biochemical analysis. A small drop of blood was collected in EDTA bulb for haematological analysis. The blood was centrifuged for plasma separation. 1.5 ml aliquots were pipetted in plastic Eppendorf tubes and were stored at -80⁰C for future analysis.

3.3.e. RECORDING OF HAEMATOLOGICAL PARAMETERS:

Haematological parameters including Haemoglobin concentration were determined by an automated cell counter.
3.3.f. ESTIMATION OF BLOOD GLUCOSE AND LIPID PROFILE:

All plasma samples were analysed in a semi-automatic biochemical analyser using commercial kits. ERBA kits supplied by Transia Biochemicals Ltd., Mumbai were used for the analysis.

3.3.f.i. Estimation of Blood Glucose Level:

The Fasting Blood Sugar (FBS) was analysed by glucose oxidase peroxidase (GPO-PAP) method, Trinder, 1969.

Principle:

Glucose in the presence of atmospheric oxygen is completely oxidised by enzyme glucose oxidase to gluconic acid and hydrogen peroxide. The hydrogen peroxide formed is broken down by peroxidase enzyme to water and oxygen. The latter oxidizes phenol which combines with amino-4-antipyrine to give a red coloured complex quinonimine. The intensity of red colour is proportional to the concentration of glucose in the sample and the intensity of colour is measured colorimetrically at 520 nm using a green filter.

Reaction:

\[
\text{Glucose oxidase} \\
\text{Glucose} + O_2 + H_2O_2 \rightarrow \text{Gluconic acid} + H_2O_2
\]

\[
\text{Peroxidase} \\
2H_2O_2 + \text{phenol} + 4 \text{Aminoantipyrine} \rightarrow \text{Quinoneimine} + 4H_2O
\]

Reagents:

1. Enzyme reagent - Consisted of Amino 4 antipyrine, glucose, peroxidase and phosphate buffer
2. Phenol (16 m mol /l)
3. Standard glucose (5.55 m mol /L )

Preparation of working enzyme reagent - The enzyme reagent was prepared in 500 ml distilled water to which 5 ml of phenol reagent was added. It was mixed gently and used after 3 hours.
Procedure:

Clean dry test tubes marked as T for test S for standard & B for blank were used. To each test tube following solutions were added in ml in given order.
Sample / standard glucose / Distilled water - 0.2 ml
Working Reagent - 3.0 ml
All the contents of the test tubes were mixed well and were incubated for 15 minutes at 37°C.
After incubation 2 ml distilled water was added, mixed well & O.D of test & standard were measured against blank using green filter (520nm)

3.3.f.ii. Estimation of Total Cholesterol level:

Total cholesterol was analysed by CHOD-PAP method. The method for this assay is based on that described by Trinder, (1969).

Principle:
Cholesterol esterase hydrolyses esters to free cholesterol and fatty acids. The free cholesterol produced as well as the preformed cholesterol is then oxidized in the presence of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. However, the quinoneimine chromogen, which has its absorption maximum at 500 nm, is produced when phenol is oxidatively coupled with 4-aminophenazone in the presence of peroxidase with hydrogen peroxide. The intensity of the final red colour produced is directly proportional to the total cholesterol concentration.

Reaction:

\[
\text{Cholesterol esterase} \quad \text{Cholesterol ester} + H_2O \rightarrow \text{Cholesterol} + \text{Fatty acids}
\]

\[
\text{Cholesterol oxidase} \quad \text{Cholesterol} + O_2 \rightarrow \text{Cholest-3-one} + H_2O_2
\]

\[
\text{Phenol peroxidase} \quad 2H_2O_2 + 4\text{-Aminoantipyridine} + \text{Phenol} \rightarrow \text{Quinoneimine} + 4H_2O
\]
3.3.f.iii. Estimation of Triglyceride level:

Triglycerides was measured by GPO-PAP Trinder method⁵.

**Principle:**

Triglycerides in the test samples are hydrolyzed by the enzyme, lipase to glycerol and fatty acids. Glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate and adenosine-5-diphosphate (ADP) in a reaction catalysed by the enzyme, glycerol kinase. The glycerol-3-phosphate produced is then converted to dihydroxy acetone phosphate (DHAP) and hydrogen peroxide (H₂O₂) by glycerophosphate oxidase. The hydrogen peroxide then reacts with 4-aminoantipyrine and 3, 5 dichloro-2-hydroxybenzene (Chlorophenol) in a reaction catalyzed by the enzyme, peroxidase to yield a red coloured quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample.

**Reaction:**

\[
\text{Lipoprotein lipase} \\
\text{Triglyceride} + H_2O \rightarrow \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{Glycerol kinase} \\
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol - 3- phosphatase} + \text{ADP}
\]

\[
\text{Glycerophosphate oxidase} \\
\text{Glycerol - 3- phosphatase} \rightarrow \text{Dihydroxyacetone phosphate} + H_2O_2
\]

\[
\text{Phenol peroxidase} \\
H_2O_2 + 4\text{-Aminoantipyrine} + p\text{-Clorophenol} \rightarrow \text{Quinoneimine} + H\text{CL} + 4H_2O
\]
3.3.f.iv. Estimation of HDL-C level:

HDL-C was measured by Phosphotungstic acid method\[^6\]. Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of Mg\(^{2+}\) ions. The cholesterol concentration in the HDL is then determined by the method described by Trinder for the assay of cholesterol.

3.3.f.v. Estimation of LDL-C level:

LDL-C was calculated by subtracting VLDL-C and HDL-C from total cholesterol. The LDL-Cholesterol concentration (LDL-C) is calculated from the total cholesterol concentration (TC), HDL-Cholesterol concentration (HDL-C) and the triglycerides concentration (TG) according to Friedewald equation (Friedewald et al., 1972)\[^7\].

\[
LDL\text{ Cholesterol (mg/dl)} = \left( Total\text{ Cholesterol} \right) - \left( \frac{Triglyceride}{5} \right) - \left( HDL\text{ Cholesterol} \right)
\]

3.3.f.vi. Estimation of VLDL-C level:

VLDL-C was calculated by indirect method as VLDL-C is one fifth of triglyceride level.
3.3. g. EVALUATION OF LIPID PEROXIDATION AND ANTIOXIDANT STATUS OF THE SUBJECTS WITH CARDIO METABOLIC RISK:

3.3.g.i. Determination of Plasma Concentration of lipid peroxidation product Malondialdehyde (MDA):

Plasma concentration of lipid peroxidation product Malondialdehyde (MDA) was estimated by the measurement of thiobarburic acid reactive substance by the method of Yagi et.al.⁸

Principle:

Malondialdehyde (MDA) levels were determined by the MDA Thiobarbituric acid (TBA) test which is the colorimetric reaction of MDA and TBA in acid solution. MDA, a secondary lipid peroxidation product, reacts with thiobarbituric acid (TBA) to generate a red coloured product, which was detected spectrophotometrically at 535 nm.

Reagents:

1. Thiobarbituric acid (TBA): 0.67 % W/V.
2. Trichloroacetic acid (TCA): 0.5% W/V.
3. n-butanol
4. Standard malondialdehyde (1,1,3,3tetraethoxypropane) - 1 ml of standard contains 0.92 g MDA (M.W. 220).

Stock solution: 15μl of standard MDA was diluted to 10 ml with double distiled water.

Working solution: 1 ml of stock solution was diluted to 100 ml with double distilled water.

Procedure:

A volume of 0.5 ml of the serum sample was added to 2.5 ml of 20% trichloroacetic acid (TCA) in a centrifuge tube.

Then 1 ml of 0.67% thiobarbituric acid (TBA) was added to the mixture. The resulting mixture was then boiled in a water bath at 100°C for 30 minutes.
The hot mixture was then allowed to cool using iced water bath.
After cooling, 4 ml of n-butanol was added to the tube and mixed using vortex mixture. The mixture was then centrifuged at 4000 rpm for 10 minutes.

The absorbance of supernatant was measured at 535 nm and the results were expressed as μmol per litre, using the extinction coefficient of $1.56 \times 10^5$ L mmol cm$^{-1}$.

Standard curve of MDA was prepared by taking different amounts of standard working solution which were equivalent to 5, 10, 15, 25 and 30 nmoles of MDA per 0.5 ml and treated similarly as described in the experiment. The obtained ODs were plotted against the nmoles of MDA.

**Calculation**:

\[
\text{Abs} = C \varepsilon L
\]
Therefore, \( C = \frac{\text{Abs}}{\varepsilon L} \)

Abs = absorbance of the test sample
C = concentration of the test sample
\( \varepsilon \) = extinction coefficient
L = light path (1 cm).
3.3.g.ii. Determination of Erythrocyte Catalase (CAT) level:

Erythrocyte catalase (CAT) was assayed colorimetrically as micromoles of hydrogen peroxide consumed per minute per milligram of Haemoglobin as described by Sinha et.al.⁹.

**Principle:**

In the ultraviolet range Hydrogen Peroxide (H₂O₂) shows a continuous increase in absorption with decrease in wavelength. The decomposition of Hydrogen Peroxide can be followed directly by decrease in extinction at 285 nm. The difference in extinction per unit time was a measure of the catalase activity.

**Reagents:**

1. Phosphate buffer [50mM; PH 7.0] - 681 mg of KH₂PO₄ was dissolved in 100 ml distilled water (Solution A) and 1.780gm of Na₂HPO₄·H₂O was dissolved in 200 ml distilled water (Solution B). Solution A and B were mixed in the proportion 1:1.

2. Hydrogen Peroxide [30mM]- 0.34 ml of 30% Hydrogen Peroxide was diluted with phosphate buffer to 100ml.

**Procedure:**

1 ml of heparinized blood was used for assay. The sample was centrifuged at 3000 rpm for 10 min and plasma was removed. Then the erythrocyte sediment in centrifuge tube was washed with normal saline for 3 to 4 time and Hb was determined.

Stock haemolysate containing 5 gm % of Hb was prepared by adding 4 parts of distilled water by volume to erythrocyte sediment.

This concentrated haemolysate was diluted with phosphate buffer and Hb content was determined by using Drabkin method¹⁰. The Drabkin's reagent contains Potassium Ferrocyanide, Potassium Cyanide and Potassium Dihydrogen Phosphate. The ferrocyanide forms methaemoglobin which is converted to cyanmethaemoglobin by cyanide.
Then, the following additions were made in two Cuvettes marked as Reference Cuvette and Test Cuvette.

1.0 ml Phosphate buffer was added in the reference cuvettes and 1.0 ml H\textsubscript{2}O\textsubscript{2} was added in sample cuvette.

To both the cuvettes 2.0 ml sample (hemolysate) was added.

The reaction was started by adding hydrogen peroxide and immediately the reading was taken at time t=0 against the reference cuvette at 285 nm.

Solution was mixed well and second reading was taken at time t=15 seconds at 285 nm.

**Calculation:**

\[
\text{Catalase activity unit/mg Hb} = [2.3/15] \frac{a}{b} [\log A1/A2]
\]

A\textsubscript{1} = Absorbance at t=0

A\textsubscript{2} = Absorbance at t=15

a = dilution factor

b = Hb content of blood or erythrocyte sediment
3.3.g.iii. Determination of Erythrocyte Superoxide Dismutase (SOD) level:

Erythrocyte superoxide dismutase (SOD) was assayed in erythrocyte lysate by modified method of Das et al.\textsuperscript{11}.

**Principle:**

Superoxide radicals generated by photo reduction of riboflavin. These radicals are allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce diazonium compound, which subsequently reacts with naphthylethylene diamine to form a red coloured azo-compound. The absorbance of coloured compound was measured at 543 nm.

**Reagents:**

1. Phosphate Buffer (pH 7.4): 1.375 gm Na2HPO4 and 1.0 gm KH2PO4 were dissolved in 500 ml of distilled water and pH was adjusted to 7.4. Finally, volume was made upto 1000 ml. with distilled water.
2. 20 mM L-Methionine: 149 mg of L-Methionine was dissolved in distilled water and diluted to 100 ml.
3. 10 mM Hydroxylamine Hydrochloride: 69 mg of Hydroxylamine Hydrochloride was dissolved in distilled water and diluted to 100 ml.
4. 50 μM Riboflavin: 1.88 mg of riboflavin was dissolved in distilled water and diluted to 100 ml.
5. Sulfanilamide: 2.5 gm. of Sulfanilamide was dissolved in 3M HCl and diluted to 250 ml.
6. N-naphthylethylenediamine: 50 mg of N- naphthylethylenediamine was dissolved in distilled water and diluted to 250 ml.
7. 1 % Triton X -100
8. 50 μM EDTA
**Procedure:**

Heparinized blood sample was centrifuged and plasma was removed. Hemolysate was prepared and treated with chloroform-ethanol mixture. The supernatant obtained was used as sample in the next step.

A set of test tubes was taken and labelled as test and control. In each test tube 1.110 ml of phosphate buffer was added.

To it, 0.075 ml L-Methionine, 0.0440 ml Triton- X 100 , 0.075 Hydroxylamine Hcl, 0.1 ml EDTA and 0.1 ml sample were added.

All the tubes were then incubated at 37°C for 10 minutes.

After this, 0.05 ml Riboflavin was added to each test tube and the test tubes were exposed to light for 10 minutes , after which 0.75 ml Sulfanimide and 0.75 ml N-Napthylenediamine were added to each test tube, and the test tubes were kept at room temperature for 20 minutes.

After 20 minutes, absorbance was read at 543 nm.

**Calculation:**

Erythrocyte superoxide dismutase activity was estimated by following formula:

One SOD Unit = Amount of enzyme required to inhibit nitrite formation of control by 50% at 37°C for 10 min.

SOD unit = (OD of Control ÷ OD of Test) – 1.
3.3.g.iv. Determination of Erythrocyte Glutathione Peroxidase (GPx) level:

Erythrocyte Glutathione peroxidase (GPx) activity was assayed in erythrocyte lysate by the modified method of Paglia and Valentine\textsuperscript{12}.

**Principle:**

GPx activity was determined by a direct spectrophotometric procedure which depends on the reaction: \( \text{GSH} + \text{H}_2\text{O}_2 = \text{H}_2\text{O} + \text{GSSG} \). The activity was determined by measuring the decrease in the absorbance of the reaction mixture at 340 nm as NADPH, \( \text{H}^+ \) was oxidized to NADP+. The decrease in the absorbance would reflect the amount of oxidized glutathione that had been formed and consequently the activity of GPx.

**Reagents:**

1. Phosphate buffer pH 7 (0.05M KH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} and 0.005M EDTA): 0.2g potassium dihydrogen phosphate, 0.5g disodium hydrogen phosphate and 0.146g ethylene diamine tetra-acetic acid was diluted in 100ml distilled water.
2. Nicotinamide adenine dinucleotide phosphate reduced form (0.0084 M-NADPH): 7mg/ml distilled water.
3. Glutathione reductase (GR): 2.3 mg/ml double water.
4. Glutathione (0.15 M GSH): 46mg/ml distilled water.
5. Sodium Azide (1.125 M NaN\textsubscript{3}): 73mg/ml distilled water.
6. Hydrogen Peroxide (0.0022 M H\textsubscript{2}O\textsubscript{2}): 0.025 ml of 30% H\textsubscript{2}O\textsubscript{2}/100 ML distilled water.

**Reaction medium:**

Recombination of the buffer and other reagents into large fresh pools allowed more expeditious assays of multiple samples with no variance in the outcome. So that to 2.58 ml phosphate buffer pH 7, the following solutions were added in turn: 0.1 ml NADPH, 0.01 ml GR, 0.01 ml NaN\textsubscript{3} and finally 0.1 ml GSH.
**Procedure:**

25 μl of sample was mixed with 0.7 ml of the reaction mixture and the enzymatic reaction was initiated by the addition of 25 μl of H_2O_2. The conversion of NADPH to NADP was followed by continuous recording of the change in absorbance of the system at 340 nm at 0, 1, 2 and 3 minutes after the initiation of reaction.

A blank (to determine the contribution of the non enzymatic oxidation of NADPH) was similarly done but the tissue fraction was replaced by an equal volume of distilled water. The reaction rate of the blank was subtracted from the experiment to determine the true enzymatic activity.

**Calculation:**

\[
A = \left[ \Delta E_{340} \text{ of sample} - \Delta E_{340} \text{ of blank} \right] \times V \times 6.22 \times n
\]

Where:

- \( A \) = enzyme activity expressed as unit per gram, where one unit is the amount of enzyme which oxidize one μmol of NADPH,H+ per minute.
- \( \Delta E_{340} \) = the change in absorbance of NADPH,H+ per minute of sample
- \( \Delta E_{340} \) = the change in absorbance of NADPH,H+ per minute of blank resulting from non enzymatic oxidation of NADPH,H+.
- \( V \) = total volume of the reagents used in the experiment and present in the cuvette.
- 6.22 = the molar absorptivity of the NADPH,H+ and it is the product of multiplication of the absorbance of 1 μmol of NADPH at 340 nm (8.34x 10^-3) by the molecular weight of NADPH,H+ (744.5).
3.3.g.v.Determination of Blood Glutathione (GSH) level:

Glutathione (GSH) in whole blood was determined by method of Butler et.al\textsuperscript{13}.

Principle:

The method depends on the reaction of the free SH-group of the reduced glutathione molecule with 5,5'-dithiobis- (2-nitrobenzoic acid) [DTNB] yielding a yellow colour product (2 nitro-5- thiobenzoic acid) that can be measured colorimetrically.

Reagents:

1- Phosphate solution(0.3M Na2HPO4): 42.6 mg disodium hydrogen phosphate / ml distilled water.
2- DTNB reagent: 40 mg DTNB/100 ml of 1 % Sodium citrate.
3- Standard GSH: Stock standard was prepared by dissolving 7.5 mg GSH in 50ml 1% metaphosphoric acid. Different concentrations were prepared by serial dilution of the stock solution to get standard solutions of 0.92, 1.86, 3.7, 7.4 and 15 mg GSH per 100 ml.

Procedure:

0.5 ml of the sample was added to 2 ml of phosphate solution, followed by the addition of 0.25 ml of DTNB reagent.

The absorbance was measured at 412 nm within 5 minutes of the addition of DTNB reagent against blank (prepared using 0.5 ml of 1% metaphosphoric acid).

Calculation:

\[
\text{GSH concentration} = \frac{\text{Absorbance of sample}}{X \text{ concentration of standard in 0.5 ml}}
\]
3.3.g.vi. Determination of Vitamin - C level:

Plasma vitamin C was estimated by DNPH method\textsuperscript{14}.

Principle:

The ascorbic acid is oxidized to diketogluconic acid in presence of strong acid solution and the diketogluconic acid reacts with 2, 4 dinitrophenyl hydrazine to form dinitrophenylhydrazone which dissolves in strong sulphuric acid solution to produce red coloured complex which can be measured colorimetrically.

Reagents:

1. 10\% Trichloroacetic acid: 10 gm of Trichloroacetic acid (TCA) was dissolved in distilled water and diluted to 100ml.
2. 2, 4 dinitrophenylhydrazine (DNPH): 2 gm of crystalline DNPH was dissolved in 100 ml of 9N sulphuric acid. (75 ml distilled water + 25 ml concentrated sulphuric acid)
3. Thiourea solution: 10 gm of thiourea was dissolved in 100 ml of 50\% ethanol and was stored in refrigerator.
4. Cupric sulphate (1.5\%): 1.5 gm of CuSO\textsubscript{4}.5H\textsubscript{2}O was dissolved in distilled water and the volume was adjusted upto 100ml.
5. Combined colour reagent: It was prepared freshly on the day of use by adding 5.0 ml of 2,4dinitrophenylhydrazine reagent + 0.1 ml of cupric sulphate solution + 0.1 ml of thiourea solution.
6. Sulphuric acid: 10.0 ml of distilled water was added in 90 ml of concentrated sulphuric acid mixed and allowed to cool. It was stored in a glass stoppered bottle in the refrigerator or a portion was cooled in ice water bath before use.
7. Stock standard (1gm \%): 1 gm of ascorbic acid was dissolved in distilled water and diluted to 100 ml.
8. Working standard (2mg \%): 0.1 ml of stock was diluted up to 50 ml with distilled water just before use.
**Procedure:**

Deproteinization: 1.0 ml of plasma was taken in a clean and dry centrifuge tube. To this 1.0 ml 10% TCA and 0.5 ml of chloroform was added and mixed well by shaking vigorously for 10 to 15 minutes and centrifuged. The clear supernatant was collected as protein free filtrate (PFF).

Colour development: 500 µL of samples and standards were taken in separate test tubes to which 100 µL of colour reagent was added.

The test tubes were incubated at 37°C for 3 hours and 750 µL 85% H₂SO₄ was added to each test tube.

The test tubes were then vortexed and kept for 30 minutes at room temperature.

The absorbance were read at 520 nm.

**Calculation:**

\[
\text{Plasma ascorbic acid} = \frac{\text{OD of Test}}{\text{OD of Standard}} \times 2 = \ldots \text{mg/dl.}
\]
3.3.g.vii. Determination of Vitamin - E level:

Serum Vitamin E (VIT-E) was measured by the method described by Jargar et.al.\textsuperscript{15}.

**Principle:**

The method was based on previous Baker and Frank method and the method of Martinel by using 2.2'-bipyridal, ferric chloride and xylene. The complex of Ferrous ions generated in this reaction with 2-2'-bipyridal was determined by using plain enzyme linked immunoabsorbent assay microplate (non-antibody coated) at 492nm.

**Reagents:**

Stock standard solution of α-tocopherol(0.27% w/v): 270 mg of α-tocopherol acetate diluted in 100 ml ethanol (aldehyde free) and mixed thoroughly.

2.2’—Bipyridyl (0.12% w/v): 120 mg 2.2'-Bipyridyl is dissolved and the volume is made upto 100 ml with n-propanol and is kept in a brown bottle.

Ferric chloride(0.12% w/v): 120 mg FeCl\textsubscript{3}.6H\textsubscript{2}O is dissolved in 100 ml ethanol and is also kept in a brown bottle.

All these solutions are stable at room temperature(24˚C ± 2˚C).

Working standard of α-tocopherol: 1 ml of stock standard solution was taken and the volume was made upto 100ml with ethanol (aldehyde free) to obtain concentration of 27μg/ml.

**Procedure:**

750 μl of ethanol (aldehyde free) and 750 μl serum were added in centrifuge tubes marked as sample.

The blank was prepared by adding 750 μl of distilled water and 750 μl of ethanol (aldehyde free). All tubes were covered tightly and shaken vigorously for 30 seconds.

Then 750 ml of xylene was added. All the tubes were again covered and shaken vigorously for another 30 seconds and then centrifuged for 10 minutes at 3000 rpm.
Xylene layer was transferred in small sized test tubes.
In each test tube, 50 μl of 2,2’-bipyridyle solution was added followed by 100 μl of ferric chloride solution and waited for 2 minutes.
The absorbance was measured at 492 nm.
The concentration of serum α-tocopherol of the sample was obtained by using standard curve prepared earlier.
4.4. EVALUATION OF CARDIO METABOLIC RISK PROFILE OF THE SUBJECTS:

Cardio metabolic risk profile of the subjects were evaluated according to consensus statement for diagnosis of general obesity, abdominal obesity and metabolic syndrome for Asian Indians\textsuperscript{16}. Presence of three or more of the following risk factors in a single women was considered of having profound cardio metabolic risk:

**Table III**: Cut offs for different cardio metabolic risk factors according to harmonized criteria\textsuperscript{16}.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Cut-Off</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased waist circumference</td>
<td>( \geq 80 ) cm.</td>
</tr>
<tr>
<td>Hypertriglyceridemia (serum TG level)</td>
<td>( \geq 150 ) mg/dl (1.7 mmol/L).</td>
</tr>
<tr>
<td>Low HDL-C</td>
<td>(&lt; 50 ) mg/dl (1.29 mmol/L).</td>
</tr>
<tr>
<td>Elevated Blood Pressure</td>
<td>SBP ( \geq 130 ) mmHg and/or DBP ( \geq 85 ) mmHg.</td>
</tr>
<tr>
<td>Hyperglycemia (fasting blood glucose level)</td>
<td>( \geq 100 ) mg/dl (5.5 mmol/L).</td>
</tr>
</tbody>
</table>

4.5. STATISTICAL ANALYSIS:

Statistical analysis was performed using SPSS 16.0 (statistical Program for the Social Sciences) for Windows. Continued variables (e.g. age) were reported as Mean \pm Standard Deviation (SD). The mean for age, height, weight, BMI, WHR and WHtR were stratified by menopausal status and ethnicity. The t-test was used to test for the difference in measurements between various groups at the level of significance P < 0.05. Pearson’s correlation coefficients between cardio metabolic risk factors and anthropometric parameters and atherogenic index were calculated. Linear regression analysis was performed to establish the relationship between anaemia, antioxidant status and cardio metabolic risk in subjects.
4.6 REFERENCES:


